Article 4

RECENT STUDIES ON MECHANISMS OF BIOACTIVATION AND DETOXIFICATION OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-BUTANONE (NNK), A TOBACCO-SPECIFIC LUNG CARCINOGEN

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Recent Studies on Mechanisms of Bioactivation and Detoxification of 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK), A Tobacco-Specific Lung Carcinogen

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ABSTRACT: This article reviews recent advances in the biochemistry and molecular biology of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific pulmonary carcinogen believed to be involved in the induction of lung cancer in smokers. Several aspects of NNK bioactivation are addressed, including identification of its metabolites in laboratory animals and humans, cytochrome P450 enzyme involvement in its metabolic activation, DNA and protein adduct formation, biological significance of the major DNA adducts formed, and mutations in oncogenes from tumors induced by NNK. Collectively, the presently available data provide a reasonably clear picture of NNK bioactivation in rodents, although there are still important gaps in our mechanistic understanding of NNK-induced tumorigenesis. The studies in rodents and primates have facilitated development of methods to assess NNK bioactivation in humans, which will be applicable to studies of lung cancer susceptibility and prevention.

KEY WORDS: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone, lung carcinogenesis, cytochromes P450, metabolic activation, glucuronidation, DNA adducts, hemoglobin adducts, K-*ras* oncogenes, O^6-methylguanine, DNA pyridyloxobutylation, DNA methylation.

I. INTRODUCTION

This review focuses on recent advances in the biochemistry and molecular biology of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Over 100 papers relevant to this topic have appeared in the past 5 years, attesting to a significant level of interest in the mechanisms by which NNK induces cancer. NNK is an important model compound for examining the mechanisms of pulmonary carcinogenesis in laboratory animals. It is also likely to play a significant role as a lung carcinogen in smokers.[1–3] Selected aspects of NNK biotransformation, adduct formation, and involvement in oncogene activation are discussed.

Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; **NNAL,** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; **NNAL-Gluc,** [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid; **7-mG,** 7-methylguanine; ***O*⁶-mG,** O^6-methylguanine; ***O*⁴-mT,** O^4-methylthymine; **HPB,** 4-hydroxy-1-(3-pyridyl)-1-butanone; **GC-MS,** gas chromatography–mass spectrometry; **NNN,** *N'*-nitrosonornicotine; **AMMN,** acetoxymethylmethylnitrosamine; **NNKOAc,** 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone.

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II. IDENTIFICATION OF NNK METABOLITES

A. Laboratory Animals

An overview of NNK biotransformation pathways is presented in Figure 1. Reduction of the NNK carbonyl group giving NNAL is a major metabolic reaction in most systems examined, both *in vitro* and *in vivo*.[4-9] Laboratory animals treated with NNK rapidly convert a significant portion of it to NNAL, which can be detected in blood minutes after injection. NNAL also is a potent pulmonary carcinogen and can be regarded as a transport form of NNK.[6,10] An important advance was the identification of NNAL-Gluc as a urinary metabolite of NNK in mice and rats.[11] The amount of the NNK dose excreted as NNAL-Gluc in the urine reached a maximum of 22% at relatively high NNK doses but was substantially lower at NNK doses closer to human exposure levels. Subsequent investigations of NNK metabolism in the patas monkey demonstrated the presence of two diastereomers of NNAL-Gluc in urine, in contrast to the rat and mouse, which had mainly one diastereomer.[9] These two NNAL-Gluc diastereomers accounted for approximately 15 to 20% of the NNK dose when the amount administered was similar to human exposure levels, suggesting the use of NNAL-Gluc as a dosimeter for human uptake of NNK. As described later, this approach has been successful and has provided some new insights into NNK metabolism in humans. NNAL-Gluc has also been established as a major metabolite of NNK excreted into bile.[12] When other pathways of NNK metabolism are inhibited, the levels of NNAL plus NNAL-Gluc in urine increase.

Major urinary metabolites of NNK in rodents and primates result from α-hydroxylation of NNK and NNAL (hydroxylation on the methylene and methyl carbons adjacent to the *N*-nitroso group), pyridine-*N*-oxidation of NNK and NNAL, and glucuronidation of NNAL (see Figure 1).[4,9,11,13] Unchanged NNK is rarely detected in urine. A minor urinary metabolite of NNK was recently identified as 6-hydroxyNNK, resulting from hydroxylation of the 6-position of the pyridine ring.[13] This is the only known pyridyl hydroxylated metabolite of NNK and, by analogy to studies on nicotine metabolism, may be formed by bacterial metabolism of NNK. Ongoing studies of NNK metabolism *in vitro* and *in vivo* in rodents and primates have failed to demonstrate significant amounts of metabolites resulting from hydroxylation β- or γ- to the *N*-nitroso group.

A surprising observation was made by Peterson et al. in a study of NNK metabolism by rat pancreatic microsomes, which were being investigated for their ability to metabolically activate NNK by α-hydroxylation.[14] Although this pathway could not be demonstrated, two new metabolites of NNK and one new metabolite of NNAL were observed. These were identified as $NADP^+$ or NADPH analogs, in which nicotinamide was replaced by either NNK or NNAL (see Figure 1). The formation of the $NADP^+$ analogs was catalyzed by NAD^+ glycohydrolase. Other pyridine compounds have been shown previously to be substrates for this reaction and its biological relevance is unclear. The NNK and NNAL $NADP^+$ analogs, which are also formed in rat liver microsomal incubations, elute from HPLC near some of the other NNK metabolites and can readily be mistaken for other metabolites if sufficient care is not taken in metabolite characterization.

A potentially important observation was the report by Murphy et al. of a glucuronide of α-hydroxymethylNNK, detected in hepatocytes and urine of phenobarbital pretreated rats dosed with NNK.[15] The isolation of this metabolite establishes conclusively that α-hydroxymethylNNK is a metabolite of NNK. This had been deduced previously from consideration of the chemistry of model compounds. More importantly, Murphy's

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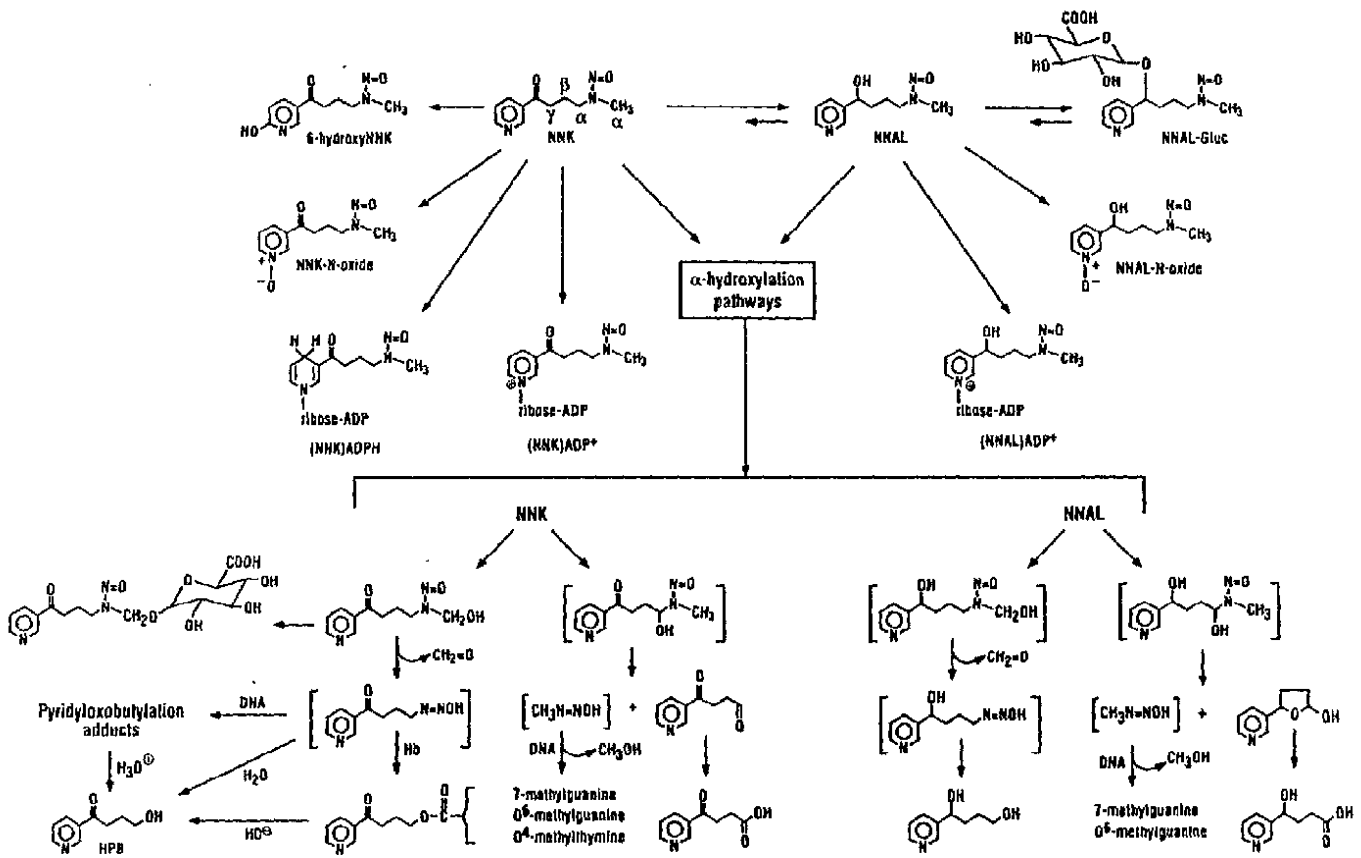

FIGURE 1. NNK metabolism pathways, as determined in rodents and primates. Compounds in brackets are postulated intermediates.

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results show that α-hydroxymethylNNK has sufficient lifetime to be converted to a stable transport form, which could be involved in the carcinogenic activity of NNK. Previously, it had been assumed that α-hydroxylated metabolites of NNK would decompose and react with cellular macromolecules at the site of their formation. α-Hydroxyglucuronides of some other nitrosamines have been observed by Wiessler and co-workers.[16]

All published data are consistent with the hypothesis that α-hydroxylation is the major metabolic activation pathway of NNK. Intermediates and products involved in this bioactivation route are summarized in Figure 1. The enzymology of α-hydroxylation and its role in adduct formation by NNK are discussed herein.

B. Humans

Based on the detection of substantial amounts of NNAL-Gluc diastereomers in patas monkey urine after treatment with low doses of NNK, methodology was developed to investigate the presence of NNAL-Gluc in human urine.[17,18] This led to the detection of both NNAL-Gluc and NNAL in the urine of smokers, but not in nonsmokers who have not been exposed passively to tobacco smoke. NNAL and NNAL-Gluc have been detected in the urine of all of the over 100 smokers analyzed to date. In one study, amounts of NNAL-Gluc ranged from 0.16 to 19.0 pmol/mg creatinine (mean ± S.D., 2.81 ± 2.92), whereas NNAL levels varied from 0.08 to 4.89 pmol/mg creatinine (mean ± S.D., 0.95 ± 1.15).[18] These levels are consistent with the amounts of NNK reported in mainstream cigarette smoke and support the proposal that uptake of NNK by a smoker results in a lifetime dose similar to that which can induce lung tumors in laboratory animals. Levels of NNAL plus NNAL-Gluc in urine are correlated with cotinine in urine.

The ratio of NNAL-Gluc to NNAL could be an indicator of detoxification potential for NNK, since NNAL-Gluc is expected to be less carcinogenic than either NNK or NNAL (although this has not been tested to date). NNAL-Gluc/NNAL ratios appear to segregate into two phenotypes, as illustrated in Figure 2.[18] Presumably, smokers in the higher ratio group would be protected to some extent from the carcinogenic effects of NNK and NNAL. The ratio phenotype is now being applied as a biomarker in molecular epidemiology studies.

NNAL and NNAL-Gluc have been identified in the urine of nonsmokers exposed to environmental tobacco smoke in a study that roughly duplicated conditions in a heavily smoke-polluted room.[19] This study established the presence of a lung carcinogen — NNAL — in the urine of nonsmokers exposed to environmental tobacco smoke, demonstrating that nonsmokers take up and metabolize NNK. The results provided experimental support for the proposal that environmental tobacco smoke can cause lung cancer. In another investigation, NNAL and NNAL-Gluc levels were quantified in the urine of people from Sudan who used an oral tobacco product — toombak — known to contain unusually high levels of NNK.[20] The NNAL and NNAL-Gluc levels in urine demonstrated that carcinogen uptake by this group was among the largest ever observed, far exceeding uptake of aflatoxin, as one example. Further analysis of this urine demonstrated that both NNAL-Gluc diastereomers were present, as seen in the patas monkey.

III. ENZYME INVOLVEMENT IN NNK BIOACTIVATION

A. Laboratory Animals

Extensive studies have now clearly shown that α-hydroxylation of NNK is catalyzed mainly by cytochromes P450.[21-34] Py-

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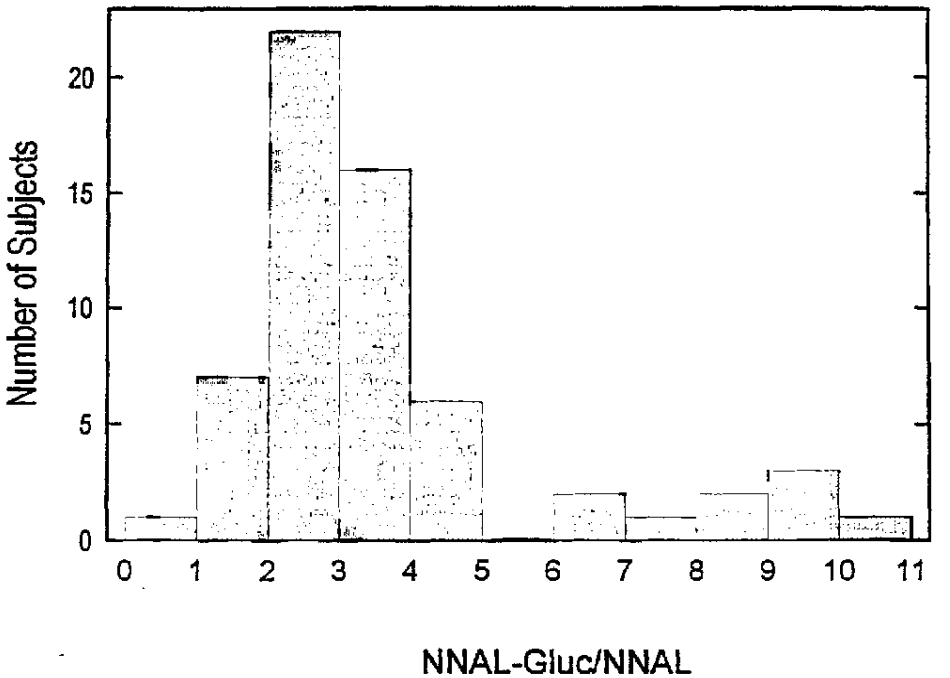

FIGURE 2. Distribution of NNAL-Gluc/NNAL ratios in the urine of 61 smokers. Each bar represents the number of subjects with ratios between 0-1, 1-2, etc.

ridine-*N*-oxidation is also mediated by cytochromes P450 in certain cases. These enzymes have generally not been implicated in the carbonyl reduction of NNK to NNAL. Studies on the role of cytochromes P450 in NNK metabolism are summarized in Table 1. Several points are noteworthy. In rat and mouse lung, catalysis of α-hydroxylation by CYP2B1 and CYP2A1, or immunochemically related forms, has been a consistent finding. CYP2B1 appears to be consistently involved mainly as a catalyst of methylene hydroxylation in the lung, whereas CYP2A1 has been implicated in both methylene and methyl hydroxylation. In liver, several forms including CYP1A2 play prominent roles. It is likely that some important cytochrome P450 activities involved in NNK bioactivation in the lung have not been characterized yet because the present evidence cannot fully account for the inhibition of NNK metabolism in lung after treatment of rodents with inhibitors such as phenethyl isothiocyanate. Several cytochromes P450 appear to play no major role in NNK metabolism in rodents. These include CYP1A1, 2C11, and 2E1. The latter is involved in the metabolism of some other nitrosamines, such as *N*-nitrosodimethylamine.[35]

B. Humans

A number of studies have examined human cytochrome P450 involvement in NNK metabolism. These are summarized in Table 1. CYP1A2 has the highest catalytic activity for methyl hydroxylation. Other studies have consistently demonstrated activity for CYP2A6 and CYP2E1.

The role of CYP2D6 is of interest because some studies have shown that exten-

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TABLE 1
Cytochrome P-450 Involvement in NNK Metabolism

Experimental System	Main P450 Involvement CYP-		No Involvement CYP-	Ref.
		A. Rat		
Lung microsomes/antibody inhibition[a]	2B	Methylene hydroxylation	4B1	21
Lung microsomes/antibody inhibition[a]	1A2[b], 2A1, 2B1	Methylene hydroxylation	1A1, 2C11, 2E1, 3A	22
	2A1	Methyl hydroxylation		
Liver microsomes/antibody inhibition[a]	1A2, 3A	Methylene hydroxylation	1A1, 2B1, 2C11, 2E1	23
	1A2, 2A1, 3A	Methyl hydroxylation		
Reconstituted system using CYP2B1	2B1	Methylene hydroxylation Methyl hydroxylation Pyridine-N-oxidation		24
Ad293 cells transfected with CYP2B1 cDNA	2B1	Pyridine-N-oxidation		25
Nasal mucosa microsomes/antibody inhibition[a]	1A2, 2A1, 3A	Methylene hydroxylation	1A1, 2B1, 2C11, 2E1	22
	1A2	Methylene hydroxylation		
		B. Mouse		
Lung microsomes/antibody inhibition[a]	2B1,2	α-Hydroxylation	1A2	26

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Lung microsomes/antibody inhibition[a]	2B1 2A1	Methylene hydroxylation	1A1, 2C11, 2E1, 1A2, 3A	27
	2A1	Methyl hydroxylation		
		C. Rabbit		
Reconsitituted system using nasal mucosa P450s	NMa	Methylene and methyl hydroxylation		28
	NMb	Methylene hydroxylation		
		D. Human		
B-Lymphoblastoid cell lines transfected with human cDNA	1A2 2A6 2D6 2E1	Metabolic activation		29
Hepatoma cells transfected with human cDNA	1A2> 2A6, 2B7, 2E1, 2F1 3A5	Methyl hydroxylation	2C8, 2C9, 2D6, 3A3, 3A4, 4B1	30
B-Lymphoblastoid cell lines transfected with human cDNA	2D6	Methylene hydroxylation Methyl hydroxylation		31
Liver microsomes/mutagenicity/antibody inhibition	2A6 2E1 1A2	Metabolic activiation	2D6, 3A4, $P450_{MP}$	32
C3H/10T1/2 cells transfected with human CYP2A6	2A6	Metabolic activation		33
C3H/10T1/2 cells transfected with human CYP2A6	2A6	Metabolic activation		34

[a] Results pertain to specific P450s or immunochemically related forms.

[b] CYP1A2 has not been detected in rat lung.

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sive metabolizers of debrisoquine are at higher risk for lung cancer. Debrisoquine metabolism is catalyzed by CYP2D6. Some controversy has arisen over the ability of CYP2D6 to catalyze NNK bioactivation. It now appears clear that CYP2D6 can metabolically activate NNK, but to a lesser extent than several other cytochromes P450.

It should be noted that NNK metabolism in human lung and liver microsomes is generally quantitatively different from that observed in rodents in that levels of carbonyl reduction to NNAL are usually greater whereas levels of α-hydroxylation are lower than in rodents. This may be an experimental artifact or result from genuine rodent-human differences. Most human lung samples obtained so far were from long-term smokers whose capacity to metabolize NNK may have been affected by years of smoking.

Although isolated cytochromes P450 can metabolically activate NNK, a prominent role of these enzymes as catalysts for NNK bioactivation in humans has not been confirmed. A recent study suggested that lipoxygenase or related enzymes may play a significant role.[36]

IV. DNA AND PROTEIN ADDUCT FORMATION

A. Laboratory Animals

As illustrated in Figure 1, metabolic activation of NNK by methylene hydroxylation or methyl hydroxylation results in methylation or pyridyloxobutylation of DNA. The presence of methyl and pyridyloxobutyl adducts in DNA of tissues from animals treated with NNK has been established conclusively.[37-41] Methyl adducts identified are 7-mG, O^6-mG, and O^4-mT. Pyridyloxobutyl adducts are quantified by measurement of HPB released by either acid or neutral thermal hydrolysis. HPB quantitation has been accomplished by radiochromatography and GC-MS. The structures of the adducts that release HPB upon acid hydrolysis have not been determined, due mainly to their instability at the nucleoside level and under conventional analytical conditions. However, HPB-releasing adducts can have a long lifetime in DNA, persisting in rat lung and liver for up to 4 weeks after a single injection of NNK.[42]

Levels of methyl adducts and pyridyloxobutyl adducts have been compared in rat lung, liver, and nasal mucosa as well as in mouse lung. In rats, the ratio of 7-mG to HPB released varied from 7 to 25 (lung) and from 13 to 49 (liver), with increasing dose.[43] The relatively greater levels of HPB at lower doses are probably due to the presence of high-affinity cytochromes P450 for methyl hydroxylation. Levels of HPB released from rat lung and liver DNA were equal to or greater than those of O^6-mG. In the nasal mucosa of rats treated chronically with NNK, levels of O^6-mG were five to seven times greater than those of HPB released.[44] In A/J mice treated with NNK. O^6-mG levels were persistent in lung for up to 15 d, and levels were consistently greater than those of HPB released.[45] The two adducts were formed and removed at different rates, consistent with differing cytochrome P450-mediated catalysis of the two metabolic activation pathways, as illustrated in Table 1.

Single-strand breaks have been observed in the DNA of rat and hamster liver after treatment with NNK, as well as in hepatocytes incubated with NNK.[46,47] Aldehydes generated in NNK metabolism via the α-hydroxylation pathway are partially involved in this mode of DNA damage.[48] Oxidative damage, as quantified by 8-oxodeoxyguanosine, has been detected in the lungs of rats and mice treated with NNK.[49]

Collectively, the available data indicate that NNK generates a wide spectrum of DNA damage in target tissues of rats and mice. Types of damage include methylation,

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pyridyloxobutylation, oxidation, and strand breakage. Some of these lesions are persistent and can cause miscoding and are probably responsible for cancer induction by NNK.

HPB-releasing hemoglobin adducts have been characterized in rats and mice treated with NNK.[50,51] One adduct is an ester, probably with aspartate or glutamate, that is hydrolyzed under mild basic conditions to release HPB.[52] The ester adduct comprises 20 to 40% of the total binding to hemoglobin; the remainder does not release HPB upon mild base hydrolysis and has not been fully characterized, although it is known that cysteine adducts are not formed to a significant extent.[53] As in DNA, the released HPB can be quantified by radiochromatography or GC–MS. Levels of HPB-releasing adducts in hemoglobin correlate with levels in DNA, although the relationship is not linear.[43] Following metabolic activation of NNK in rat hepatocytes, the α-hydroxynitrosamine or its transport form can migrate out of the hepatocyte and into the red blood cell, where it reacts with hemoglobin.[54] Hemoglobin itself also can activate NNK by methylene hydroxylation, resulting in methylation of the protein, but not in the formation of HPB-releasing adducts.[54] The HPB-releasing adduct has proven to be a useful indicator of extents of metabolic activation of NNK in studies with laboratory animals.

B. Humans

Two studies have reported significant differences between smokers and non-smokers in levels of 7-mG. In one, mean levels of 7-mG were 6.9/10^7 nucleotides in total white blood cells, 4.7 in granulocytes, and 23.6 in lymphocytes of smokers compared with values of 3.4, 2.8, and 13.5/10^7 nucleotides in nonsmokers.[55] A second study analyzed 7-mG in bronchial DNA samples and found mean levels of 17.3 adducts per 10^7 nucleotides in smokers and 4.7 in nonsmokers.[56] Levels of O^6-mG in placental DNA did not differ between smokers and non-smokers.[57]

HPB-releasing adducts in human lung and trachea were quantified by GC-MS.[58] Adducts were higher in smokers than in nonsmokers, with levels of HPB-releasing adducts ranging from 0.004 to 0.072 pmol/μmol guanine. These adducts could be produced from NNN as well as NNK.

Although the presently available data indicate that NNK produces DNA adducts in human lung, additional studies are necessary to investigate the relationship of adduct levels to smoking patterns, individual metabolic characteristics, mutations, and cancer. This approach is hindered to some extent by the difficulty in obtaining lung DNA from humans.

The quantitation of hemoglobin adducts thus presents an attractive alternative because hemoglobin is readily available. A GC-MS method for quantifying HPB-releasing adducts from human hemoglobin has been standardized.[59,60] HPB-releasing adducts have been quantified in smokers, snuff-dippers, and nonsmokers. The earlier work has been reviewed.[61] In two recent studies, levels of HPB-releasing hemoglobin adducts were quantified in nasal snuff users from Germany and in Sudanese toombak users.[20,62] Both groups showed elevated levels of hemoglobin adducts, similar to the earlier finding of elevated adduct levels in snuff-dippers from the U.S. In smokers, HPB-releasing adducts are elevated above background in only a subset of individuals and appear to be generally lower than in snuff users. This may relate to patterns of enzyme induction or inhibition due to constituents of tobacco smoke, but this requires further investigation.

V. BIOLOGICAL SIGNIFICANCE OF DNA METHYLATION AND PYRIDYLOXOBUTYLATION

The well-established miscoding properties of O^6-mG and the availability of sensi-

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tive immunochemical and HPLC-fluorescence methods for its quantitation encouraged fairly extensive investigation of its role in tumor induction by NNK following its initial identification in DNA of NNK-treated rodents.[37] Studies on the potential role of HPB-releasing DNA adducts have lagged behind since the requisite radiochemical or GC-MS methodology was developed later. Presently, it appears that both methylation and pyridyloxobutylation of DNA play some role in tumor induction by NNK in rats and mice.

Belinsky and co-workers carried out extensive investigations of the role of O^6-mG in lung tumor induction by NNK in rats.[63] They discovered that the Clara cells of the rat lung have a high capacity to activate NNK, presumably via a high-affinity cytochrome P450 enzyme, resulting in formation of substantial amounts of O^6-mG compared to other cell types of the lung. As Clara cells appear to have relatively low levels of the O^6-mG-DNA methyltransferase repair enzyme,[64] the adducts accumulated and persisted. A dose-response study of NNK-induced lung tumorigenesis demonstrated that there was a linear correlation between lung tumor induction at various doses and levels of O^6-mG in the Clara cells of the rat lung.[63] These results strongly suggest that O^6-mG is important in lung tumor induction by NNK. However, the tumors appear to arise mainly from the alveolar type II cells. These cells also metabolically activate NNK, but the linear correlation with tumor induction seen for O^6-mG in Clara cells was lacking. This apparent contradiction has not been resolved. However, it should be noted that carcinogenicity studies of [methylene-D_2]NNK and [methyl-D_3]NNK did not show any difference vs. NNK lung carcinogenicity in rats.[65] If methylation alone were important, we would have expected lower carcinogenicity of [methylene-D_2]NNK than NNK. It is likely that both methylation and pyridyloxobutylation of DNA are important in lung tumorigenesis by NNK. Pyridyloxobutyl DNA adducts are highly mutagenic in several bacterial and mammalian test systems, with activity sometimes exceeding that of methyl adducts.[66,67] Pyridyloxobutylating compounds that do not methylate, such as NNN and NNKOAc, are carcinogenic, although generally less so than are methylating agents. An interesting property of pyridyloxobutylated DNA is its ability to inhibit O^6-mG-DNA methyltransferase; in this way it can act as a cocarcinogen with a methylating carcinogen.[68]

Belinsky and co-workers proposed that a combination of O^6-mG adducts and toxicity was important in the induction of nasal tumors in rats by NNK.[69] We also investigated this question by carrying out comparative studies of [methylene-D_2]NNK, [methyl-D_3]NNK, and NNK with respect to DNA adduct formation in the olfactory and respiratory portions of the nasal mucosa.[44] Bioassays had shown that [methylene-D_2]NNK was a stronger nasal carcinogen than NNK in the rat, whereas the activity of [methyl-D_3]NNK was similar to that of NNK. In the rats treated with [methylene-D_2]NNK, levels of O^6-mG in DNA from both the olfactory and respiratory portions of the nasal mucosa were significantly lower and levels of HPB-releasing DNA adducts higher than in the rats treated with equivalent doses of the less carcinogenic compounds NNK or [methyl-D_3]NNK. HPB-releasing DNA adducts also were formed in the nasal mucosa of rats treated with the nasal carcinogen NNN, which cannot methylate DNA. These results strongly support the role of DNA pyridyloxobutylation in rat nasal carcinogenesis by NNK.

Liu et al. studied the formation and persistence of O^6-mG in the livers of rats or hamsters treated with NNK.[70] High doses of NNK induce liver tumors in rats but not in hamsters. They found that O^6-mG persisted in hamster liver, but not in rat liver, due to more effective depletion of O^6-mG-DNA methyltransferase in the former. These results do not support the role of O^6-mG in

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liver tumor induction by NNK in rats and suggest that other factors are involved. An immunochemical investigation of O^6-mG localization in various tissues of rats, mice, and hamsters treated with NNK was carried out by Van Bentham et al., who also concluded that factors other than O^6-mG must be important in the induction of tumors by NNK.[71]

In contrast to the results discussed, a relatively consistent picture has emerged in studies of DNA alkylation by NNK and lung tumor induction in the A/J mouse. Comparative tumorigenicity studies of [methylene-D_2]NNK, [methyl-D_3]NNK, and NNK demonstrated that [methylene-D_2]NNK was clearly less active than the other two compounds; levels of O^6-mG induced by this compound also were lower than those of the other two compounds.[72] An extensive comparative study of DNA adduct and tumor formation by NNK, AMMN (a DNA methylating agent), and NNKOAc (a DNA pyridyloxobutylating agent) in A/J mice clearly demonstrated the importance of persistence of O^6-mG in lung tumor formation.[45] Other studies have demonstrated the presence of O^6-mG in the alveolar type II cells, believed to be the precursor cells for adenomas and adenocarcinomas induced by NNK in A/J mice.[73] Analysis of mutations in the K-*ras* gene isolated from these tumors has shown a high prevalence of G to A transitions in the second base of codon 12, consistent with the expected mutation induced by O^6-mG.[73]

Collectively, the presently available results favor an important role for O^6-mG in tumor induction by NNK in the A/J mouse; studies in rats uniformly indicate that other factors, including DNA pyridyloxobutylation, are critical. Oxidative damage to DNA and single-strand breaks also may be involved in tumor induction by NNK. Although this discussion has focused on DNA damage and its role in carcinogenesis, other factors are clearly involved. Lung tumor induction by NNK in the C3H mouse resulted in *ras* activation, as in the A/J mouse, yet the tumors were smaller and less numerous than in the A/J mouse.[74] In the C57BL/6 mouse, another "resistant" strain, O^6-mG formation by NNK was similar to that observed in the A/J mouse, but K-*ras* activation and tumor formation were less prevalent.[75]

VI. MUTATIONS IN ONCOGENES FROM NNK-INDUCED TUMORS

Following the initial studies of Belinsky et al. on activation of the K-*ras* oncogene in lung tumors induced by NNK in A/J mice,[76] a number of other studies have appeared, as summarized in Table 2. All studies have found that, when the K-*ras* gene is activated in lung tumors induced by NNK, the main activating mutation is GGT → GAT in codon 12. Several points are of interest in Table 2. Whereas activated K-*ras* genes have been detected in lung tumors from A/J mice and Syrian golden hamsters, they have not been found in lung tumors induced by NNK in F-344 rats. This suggests that pathways independent of K-*ras* activation are important in lung tumor induction by NNK in rats.

The presence of a GGT → GAT mutation in activated K-*ras* genes is consistent with the hypothesis that it results from direct reaction of the NNK-derived methylating agent with the gene, resulting in O^6-mG, which causes the observed G to A transition mutation. The pyridyloxobutylating agent NNKOAc induced both G to A transitions and G to T transversions in lung tumors from A/J mice, in contrast to the methylating agent AMMN, which caused exclusively G to A transitions, similar to NNK. These data also support the prominent role of DNA methylation in A/J mouse lung tumor induction by NNK.

GGT → GAT mutations in codon 12 have also been observed in hyperplasias induced by NNK in A/J mouse lung, consistent with the proposal that O^6-mG causes them. Other aspects are not as clear. For example, tumors from mice treated with NNK

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TABLE 2
Mutation Induction by NNK and Related Compounds in K-*ras* and p53 Genes from Lung Tumors

Compound	Species	K-*ras*	p53	Ref.
NNK	A/J mouse	GGT → GAT, codon 12 (15/20)[a] — hyperplasias GGT → GAT, codon 12 (7/10) — adenocarcinoma		73, 76
NNK	A/J mouse	GGT → GAT, codon 12 (26/28)		77
AMMN	A/J mouse	GGT → GAT, codon 12 (18/18)		
NNKOAc	A/J mouse	GGT → GAT, codon 12 (8/21) GGT → TGT, codon 12 (5/21) GGT → GTT, codon 12 (4/21)		
NNK	A/J mouse	GGT → GAT, codon 12 (19/19) GGT → GAT, codon 12 (11/34) — promotion by butylated hydroxytoluene		78
NNK	A/J mouse		Not detected	Ronai et al. unpublished
NNK	C3H mouse	GGT → GAT, codon 12 (11/11)		74
NNK	B6C3F1 mouse	GGT → GAT, codon 12 (46/59)	Not detected	79
NNK	C57BL/6 mouse	GGT → GAT, codon 12 (1/22) CAA → CGA, codon 12 (1/22)	Not detected	75
NNK	Syrian golden hamster	GGT → GAT, codon 12 (21/35)	Not detected	80
NNK	F344 rat	Not detected		81

[a] Numbers in parentheses: number of tumors having mutation/number of tumors analyzed.

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and butylated hydroxytoluene, which promotes lung tumor development, had a lower frequency of K-*ras* mutations than did those in mice treated with NNK alone. In C3H mice, K-*ras* activation was seen in all tumors analyzed, but the tumors were not as numerous or aggressive as those in A/J mice, indicating that other factors are involved. K-*ras* activation was infrequent in C57BL/6 mouse lung tumors induced by NNK.

No studies have identified mutations in the p53 tumor suppressor gene in lung tumors induced in rodents by NNK.

In humans, activated K-*ras* genes with mutations in codon 12 have been detected in 16% of lung tumors and in 24% of adenocarcinomas of the lung.[82] The frequency of mutations is GGT → TGT (58%), → GTT (16%), and → GAT (19%). If the A/J mouse or Syrian golden hamster were the perfect model for human lung cancer, one could conclude perhaps that most of the observed mutations were due to compounds other than NNK. In the A/J mouse model, the methylation pathway is very important, but its relative importance in humans is unclear at present. If pyridyloxobutylation is important in human lung tumor induction, one might expect a larger proportion of G to T transversion mutations, as seen with NNKOAc. These aspects require further study. Presently, it is premature to attempt to relate mutational data from lung tumors induced in humans by cigarette smoke to data obtained in laboratory animals with individual constituents of smoke.

VII. CONCLUSIONS

The lung carcinogenicity of NNK in rats was first reported in 1980 and the first metabolism study also appeared that year.[4,83] In the last 14 years, a large number of studies have resulted in a fairly clear picture of NNK bioactivation, detoxification, and tumor induction mechanisms. NNK is metabolically activated by cytochrome P450 enzymes in rodent tissues. Human cytochromes P450 also can activate NNK, but their quantitative role is still unclear. The metabolic activation of NNK, by α-hydroxylation results in methylation and pyridyloxobutylation of DNA. There are competing partial detoxification reactions, including pyridine-*N*-oxidation and glucuronidation of NNAL. The DNA methylation and pyridyloxobutylation pathways are crucial in tumor induction by NNK and, depending on the species and tissue involved, one or the other pathway or both may have important roles. The adducts formed in these reactions can activate the K-*ras* oncogene in mice and hamsters, but this has not been observed in rats.

The mechanism by which NNK selectively induces lung tumors in rodents is not crystal clear, but several aspects favor this result: (1) rodent lung contains cytochromes P450 that efficiently metabolically activate NNK by α-hydroxylation, resulting in DNA binding; (2) the DNA adducts formed, such as O^6-mG and HPB-releasing adducts, can be highly persistent in lung or in particular cell types due to depletion of critical repair enzymes; and (3) these methyl and pyridyloxobutyl DNA adducts are mutagenic, leading to permanent alterations in important genes.

In parallel with DNA adduct formation in the lung, NNK also pyridyloxobutylates hemoglobin. The resulting adducts can be used to estimate the extent of NNK bioactivation under a given set of conditions, and possibly to estimate risk for tumor development. The ratio of NNAL-Gluc to NNAL in urine, as well as the total amount of these metabolites excreted, can also indicate the extent to which NNK is metabolically activated. The hemoglobin adducts and urinary metabolites of NNK are measurable in smokers and such biomarkers may be useful in extending our understanding of mechanisms of lung tumor induction in humans.

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